



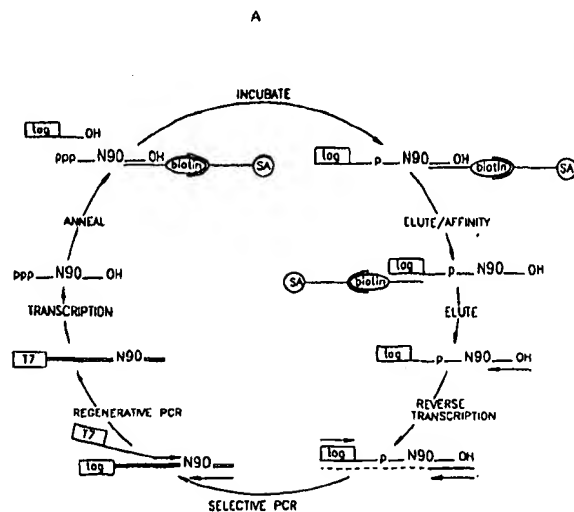
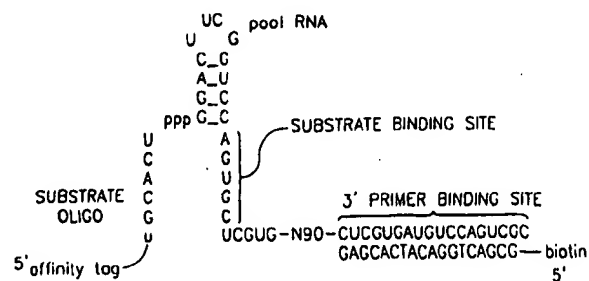
## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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(21) International Application Number: PCT/IL99/00557 (22) International Filing Date: 22 October 1999 (22.10.99) (30) Priority Data: 126731 23 October 1998 (23.10.98) IL (71) Applicant (for all designated States except US): INTELLIGENE LTD. [IL/IL]; P.O. Box 35241, 91351 Jerusalem (IL). (72) Inventors; and (75) Inventors/Applicants (for US only): NATHAN, Asher [IL/IL]; Reuven 47, 99000 Bet Shemesh (IL). ELLINGTON, Andy [US/US]; 2421 N. Dunn, Bloomington, IN 47401 (US). (74) Agent: REINHOLD COHN AND PARTNERS; P.O. Box 4060, 61040 Tel Aviv (IL).		(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> Without international search report and to be republished upon receipt of that report.	

(54) Title: DETECTION OF ANALYTES

## (57) Abstract

The invention concerns a method where catalytic nucleic acid sequence in the presence of an analyte (which may be a nucleic acid sequence or a non-nucleic acid sequence) converts a substrate to a catalytic product. The catalytic product may then be amplified by state of the art amplification methods such as PCR, LCR, 3SR and NASBA.



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## DETECTION OF ANALYTES

### FIELD OF THE INVENTION

The present invention concerns methods for detection of analytes in a sample and kit for use in said methods. More specifically, the invention concerns methods where the signal is amplified using conventional amplification  
5 technologies such as RT-PCR.

### BACKGROUND OF THE INVENTION

Ribozymes have been shown to mirror many of the catalytic properties of their protein counterparts, including the ability to be regulated by specific effector molecules. The hammerhead ribozyme has been designed to act as an allosteric  
10 ribozyme with a nucleic acid effector<sup>(1)</sup> and, more recently, aptamers grafted to the hammerhead ribozyme have served as allosteric sites for non-nucleic acid effectors<sup>(2)</sup>. The rational design of chimeric 'aptazymes' has proven to be remarkably facile, and aptazymes that can sense analytes such as ATP, FMN, and theophylline have been created<sup>(2-5)</sup>. However, most allosteric ribozymes which  
15 have been designed have been activated only 10- to 100-fold by their effectors, hampering their potential use in biotechnology or diagnostic applications.

Another approach for designing ribozymes having desired activity is by using what is termed as *in vitro* evolution, a method involving repetitive cycles of selection of ribozymes from a pool of sequences under specific conditions and  
20 amplification of the selected sequences. Ribozymes selected from random sequence populations have been shown to catalyze a variety of chemical reactions, to exhibit exquisite specificities for their substrates, and to turnover substrates at rates up to 100/minute<sup>(6-8)</sup>. It is likely that regulatory mechanisms such as allostery should also

arise as a consequence of evolution for catalytic function. For example, natural ribozymes such as the Group I and Group II self-splicing introns, have evolved to regulate successive, transesterification steps by undergoing programmed conformational transitions<sup>(9-12)</sup>.

5 Several methods are known in the art for amplification of nucleic acid sequences, the best known are PCR, LCR, 3SR and NASBA. While these methods dramatically increase the number of nucleic acid sequences in the sample thus creating a detectable signal, not all nucleic acid sequences can be amplified using these techniques for various reasons such as nucleic acid sequences which have a  
10 GC-rich region and a complicated secondary structure which hinders their amplification by state of the art amplification techniques. In addition, these amplification techniques are suitable only for the detection of nucleic acid sequences and not for detection of molecules other than nucleic acids such as for the detection of proteins. Non-nucleic acid molecules are routinely detected by  
15 immunoassays and where the analyte is in minute quantities a large effort is required in order to produce a detectable signal.

It would have been highly desirable to provide a method for detection of analytes in a sample which would be able, on the one hand, to detect all nucleic acid sequences even those which have complicated secondary structures (or even  
20 detecting the secondary structures themselves), and on the other hand, would be able also to detect non-nucleic acid molecules while producing a significant detectable signal.

## SUMMARY OF THE INVENTION

The present invention is based on the realization that it is possible to use  
25 allosteric nucleic acid sequences, which become catalytically active only in the presence of analytes, so that the product of the catalytic activity of these sequences is produced only in the presence of an analyte, and then amplify said product, by state of the art nucleic acid amplification mechanisms such as PCR, LCR, NASBA and 3SR.

In accordance with the present invention nucleic acid sequences are used which produce a product only in the presence of the analyte and the product is amplified. By state of the art amplification techniques. Thus the allosteric nucleic acid sequences and the products produced by their catalytic activity serve as  
5 mediators between the analyte and the amplification machinery.

Thus, the present invention concerns a method for the detection of an analyte in a sample comprising:

- (i) providing a nucleic acid sequence which is initially essentially catalytically inactive, and which becomes catalytically active in the  
10 presence of the analyte;
- (ii) providing a nucleic acid substrate for the catalytic activity of the nucleic acid sequence;
- (iii) contacting the nucleic acid sequence and the substrate with the sample under conditions allowing catalytic activity of nucleic acid  
15 sequences, thereby allowing, in the presence of the analyte, catalytic activity of the nucleic acid sequence on the substrate to produce a catalytic product which is a nucleic acid molecule;
- (iv) contacting the catalytic product with a nucleic acid amplification system capable of multiplying the number of copies of the catalytic  
20 product, a significant increase in the number of copies of the catalytic product indicating the presence of the analyte in the sample.

The detection of the analyte may be qualitative, i.e. giving yes/no binary answer to the presence of analyte, or may be quantitative, giving an indication of the amount of analyte in the sample as will be explained hereinbelow.

25 The analyte may be a nucleic acid sequence, such as a cDNA or RNA sequence indicative of the presence of a mutation, the presence of an infectious organism, etc. As indicated above the nucleic acid analyte may be in the form which is unsuitable for direct amplification for example due to secondary structure which can hinder direct amplification for example by PCR. Alternatively, the  
30 analyte may be a non-nucleic acid analyte. Examples of non-nucleic acid analytes

are proteins, peptides, glycoproteins, glycopeptides, membranal fragments, phosphorylated nucleosides, hormones, drugs, organic or non-organic contaminants, noxious agents, etc.

The sample may be any liquid sample in which the analyte should be determined, examples of which are blood, serum, urine, water (for the detection of contaminants or infectious agents), milk, suspension of food, soils, etc. (for the detection of contaminants, noxious agents or infectious agents).

The nucleic acid sequence, may be composed of any type of nucleotides, i.e. may be composed of rNTPs, dNTPs, a mixture of rNTPs and dNTPs, and may include also some non-naturally occurring nucleotides. By a preferable embodiment, the nucleic acid sequence is composed of rNTPs, i.e. is a ribozyme, or as will be termed hereinafter at times "*an allosteric ribozyme*".

In the absence of the analyte, the catalytic nucleic acid sequence is essentially inactive, i.e. has either no catalytic activity, or has a very low level of catalytic activity, which increases in the presence of the analyte.

Once in the presence of analytes, the catalytic activity of the nucleic acid sequence is initiated, or substantially up-regulated. The analyte may induce such inactivity by a number of different mechanisms. By one option, for example as specified in Porta *et. al* (*Bio/Technology*, 13, 161-164, (1996)) (in connection with a nucleic acid allosteric effector) and in WO 94 13833 (in connection a non-nucleic acid allosteric effector), the analyte may induce a conformational change in the nucleic acid sequence. For example, the nucleic acid sequence may contain a portion which at its native state ( i.e. in the absence of the effector), masks the catalytic region of the nucleic acid sequence, thus rendering it inactive. Once in the presence of the analyte, this inhibitory sequence binds to the analyte, changing its conformational change and unmasking the catalytic region, thus relieving the nucleic acid sequence from its inhibitory constraint and allowing it to become catalytically active.

By another option, as indicated in commonly owned international application WO 98/08974 the effector (which may be a nucleic acid sequence or

non-nucleic acid molecule) provides a missing component which is required for catalytic activity of the nucleic acid sequence (termed: "*protonucleozyme*"), not necessarily by conformational change of the latter but by other mechanisms. For example, where the effector is a nucleic acid sequence, it may complete a missing  
5 segment of one or more nucleotides in the *protonucleozyme* thus rendering it active. Where the effector is a non-nucleic acid molecule it can provide the *protonucleozyme* with a component required for its catalytic activity thus rendering it active.

The method of the invention also comprises a step of providing a substrate  
10 for the catalytic activity of the nucleic acid sequence. While it is known that catalytic nucleic acid sequences can act also on non-nucleic acid sequences as substrates in the context of the present invention, the substrate, and consequently the catalytic product as will be explained below, have to be nucleic acid sequence in order to be amplified at later stages

15 The substrate nucleic acid sequence, may be a separate sequence than the nucleic acid sequence of step (i), and then when the nucleic acid sequence is catalytically active, it acts in *trans* on the substrate. Alternatively, the substrate may be a part of the nucleic acid sequence, and in that case the nucleic acid sequence acts in *cis*.

20 The catalytic activity of the catalytically active nucleic acid sequence, should be such which can produce a nucleic acid catalytic product, capable of being amplified by various amplification systems. This means that the catalytic activity, converts the substrate, which is in a form not suitable for amplification, into a catalytic product which is in a form suitable for amplification.

25 For example, the catalytic activity may be ligation, and in that case can bring together two substrate nucleic acid molecules, thus forming together a suitable template for PCR, capable of hybridizing with two PCR primers present in the detection reagents.

By another embodiment, the substrate may have a sequence which, due to  
30 some secondary structure such as folding or hybridization renders the substrate

unsuitable for PCR amplification. In that case, the catalytic activity may be cleavage, (where said secondary structure is at one terminal of the substrate) or splicing out (where this region is at the middle of the substrate), thus forming a suitable template for amplification. By another substrate the template may have a primer-binding region which is split to two parts separated from each other by an additional sequence. Only upon removal of the additional sequence by splicing is a correct primer binding site formed in the product and the catalytic product can be amplified.

Another option, is a substrate which is immobilized in such a manner in which it is not accessible to primers for amplification, such as primers required for PCR amplification. In such a case, cleavage or splicing out by the catalytically active nucleic acid sequence, can convert the substrate to a catalytic product. The term "*catalytic product*" refers to a substrate which has been catalytically modified in such a way which renders it suitable for amplification.

Amplification systems may be PCR, LCR, 3RS, and NASBA.

The method of the present invention, is not only suitable for detection of the presence of an analyte in a sample, but also for determination, or estimation, of the amount of the analyte in the sample.

This is done by preparing a calibration scale for known amounts of analytes, wherein the catalytic product for each known amount of analyte is amplified under predetermined conditions. Then, the sample is brought into contact both with the allosteric nucleic acid sequence of the invention, and the amplification system, and the amount of amplified catalytic product is compared with the calibration scale, in order to determine the amount of analyte initially present in the sample.

The present invention, features some advantages which make it very attractive for use.

First, it is essentially the only method which enables detection of non-nucleic acid analytes, using a nucleic acid amplification system such as PCR, LCR, etc. In fact, the method of the present invention transforms, by use of the catalytic activity of the nucleic acid sequence, a signal supplied by a non-nucleic acid



analyte, to a signal (i.e. the catalytic product) which can be read and amplified by a nucleic acid amplification system.

In addition, there are some cases, where direct amplification of nucleic acid sequences by PCR, LCR, etc. is not possible due to secondary structural constraints. In such a case, the sequence which serves as an analyte, although not suitable for amplification, can serve as an allosteric effector for the nucleic acid sequence of the invention, which can in fact "*transform it*" by mediation of the catalytic product, to a form suitable for amplification by amplification systems.

Finally if the allosteric nucleic acid sequence used is a DNA sequence than it is possible to immobilize these sequences on solid support and use several rinsing steps and thus increase the sensitivity of the method.

## BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A shows the design ribozyme N90 design and selection scheme. Pool RNA substrate binding site, substrate oligo and primer binding sites are all indicated.

Fig. 1B shows the selection scheme used to isolate ligase ribozymes from the N90 pool;

Fig. 2A shows proposed secondary structural models for active and conformations of the L1 ribozyme and Fig. 2B shows proposed conformations for inactive ribozyme. The effector oligonucleotide is italicized. Substrate:ribozyme pairs that were examined for activity are shown adjacent to the substrate-binding site in Fig. 2A;

Fig. 3 shows nuclease probing of the L1 ribozyme. RNase A or RNase T1 digests were carried out in either the presence (+) or absence (-) of the wild-type substrate and/or effector described in the "*Detailed Description*". The sizes of bands were determined in relation to standards of the same sequence (not shown). Alkaline hydrolysis (ah) ladders allow the nucleotide differences between bands to be determined. Nucleotides listed along the sides of the autoradiogram indicate positions that show differential cleavage in the presence orange or

absence green of the oligonucleotide effector. Digestions under denaturing conditions (denat) reveal most of the structurally sequestered sites on the ribozyme;

Fig. 4A shows detection of an oligonucleotide effector. Internally labeled L1 ribozyme was mixed with a substrate in the presence of varying concentrations of its cognate oligonucleotide effector, and ligated and unligated RNAs were separated by gel electrophoresis and quantified. Ligation products can be detected down to 10 nM effector. Fig. 4B shows detection of an oligonucleotide effector by RT-PCR. Reactions similar to those described in Fig. 4A were carried out, but the ligation products were amplified by PCR in the presence of a radiolabeled primer prior to detection. "Background" is a no template control for RT-PCR, and a time zero reaction for ligation. ;

Fig. 5 shows detection of a non-oligonucleotide effector. Fig. 5A shows adenosine aptazyme design. Some residues are an anti-adenosine aptamer<sup>(23)</sup> grafted onto the L1 ribozyme in place of non-conserved residues to generate L1-ATP. Primer-binding regions are in bold. Fig. 5B shows detection of a non-oligonucleotide effector by ligation. Reactions similar to those described in Fig. 4A were carried out in the presence of varying concentrations of ATP and quantitated. Fig. 5C shows optimization of an adenosine aptazyme. The adenosine aptazyme L1.dB2-ATPm1 was generated by truncation and sequence substitution (shown in blue) of L1-ATP. The activation of L1.dB2-ATPm1 in the presence of 1 mM ATP improved from 30-fold to 800-fold; and

Fig 6. shows a schematic representation of the method of the invention.

## 25 DETAILED DESCRIPTION OF SPECIFIC EMBODIMENTS

Fig. 6, shows a schematic representation of the method of the invention with several modes of catalytic activity.

The analyte 1 is a protein which presence should be determined in a sample. The sample containing analyte 1, is brought into contact with a nucleic acid sequence 2 having initially no catalytic activity. The binding of analyte 1 to nucleic

acid sequence 2, gives rise to a catalytically active nucleic acid-analyte complex 3 which features catalytic activity on the substrate. Three different options are shown. In the first option (left), substrate 4 shows a PCR template, having regions appropriate for primer 1 (P1) and for primer 2 (P2), the template being broken into  
5 two parts. The complex nucleic acid-analyte 3 is capable of ligating to the two substrates 4 to give rise to catalytic product 5.

By another option (middle), the substrate 4' is incapable of amplification, since in the proximity of its primer 1 binding site (P'1), is a double-stranded region serving as a secondary structure constraint due to presence of extra sequence 6. In  
10 that case, the catalytic activity of the complex 3 is cleavage. Once the extra strand 6 of substrate 4' is cleaved, a suitable template for PCR amplification 5 is produced.

By a third option (right), substrate 4'' has primer 1 broken into two parts (shown schematically as  $P1''_{1/2}$  and  $P1''_{1/2}$ ) separated by an extra sequence 6'. The catalytic activity of complex 3 in that place is splicing, and upon splicing extra  
15 sequence 6' is spliced out, and a suitable template 5 is formed. Template 5 of any of these options is in fact the "catalytic product" and in the presence of suitable reagents for PCR, can be amplified to give a detectable amount of copies, and even quantitize. For quantitization purposes, nucleic acid sequence 2, should be in an access to analyte 1. Thus, the amount of complexes 3 which will be formed, will be  
20 directly proportional to the amount of analyte 1 produced..

Under controlled condition, the amount of product 5 produced by catalytic activity on substrate 4, 4' or 4'' will be again proportional to the number of complex 3, and of course the amount of amplified product 5 by PCR will again be proportional to the amount of original product 5 produced.

25 The said amount of amplified product 5 can be compared to a calibrated scale prepared beforehand, which correlate known amounts of analyte, with the amount of amplified product 5 produced by PCR.

## **Experimental Procedures**

### **A. Ribozyme N90 pool construction**

The pool contained a central randomized region 90 nt in length (N90) flanked on both sides by constant sequence regions (5' CTTCGGTCCAGTGCTCGTG-N90-CTCGTGATGTCCAGTCGC 3'). The randomized region was drawn from a mixture containing a 3:3:2:2 ratio of dA:dC:dG:dT phosphoramidites to avoid the preferential incorporation of dG and dT<sup>(13)</sup>. A roughly equal representation of nucleotides (27:28:23:22) was verified by dideoxy sequencing of 12 N90 clones. DNA corresponding to the full-length N90 pool was isolated from a 8% denaturing polyacrylamide gel. The yield of the purified oligonucleotide was 13.8 nmol, but only 15% of these molecules were sufficiently free of chemical lesions to allow primer extension by Taq polymerase. The pool was amplified in a 167 mL PCR reaction using primers that extended the 5' constant region and added the promoter sequence for T7 RNA polymerase (5' TTCTAATACGACTCACTATAGGACTTCGGTCCAGTGCTCGTG and 5' GCGACTGGACATCACGAG, T7 promoter underlined). The final complexity of the population should have been ca.  $1.2 \times 10^{15}$ , and one pool equivalent was transcribed with T7 RNA polymerase (Epicentre, Madison, WI) in a 1.5 mL reaction and then purified on a 6% denaturing polyacrylamide gel.

### **B. N90 selection for catalysis.**

Starting with an input of  $1.8 \times 10^{15}$  N90 RNA molecules, five rounds of selection were performed according to the procedure of Bartel and Szostak<sup>13</sup> with primers and substrate oligonucleotides specific for the N90 pool. In short, pool RNA was annealed to a biotinylated DNA primer complementary to its 3' constant region (5' biotin- GCGACTGGACATCACGAG) and captured on streptavidin-agarose (Gibco BRL, Gaithersburg, MD). The beads were washed and immersed in selection buffer (30 mM Tris pH 7.4, 600 mM NaCl, 1 mM EDTA, 0.1% NP40) and MgCl<sub>2</sub> added to a final concentration of 60 mM.

Following a 10 minute equilibration at room temperature, the reaction was initiated by addition of a two-fold molar excess of substrate oligonucleotide (5' tag-UGCACU where tag = dA<sub>22</sub>, dT<sub>22</sub>, or TGATCGCTTGATCGCTTGATCG; RNA in bold). Rounds 1 through 4 were incubated for 16 hours with cycling  
 5 between 25° and 37°C. Round 5 was incubated 2 hours at 25°C before the reaction was stopped by addition of EDTA. RNA was then eluted from the streptavidin with base, neutralized, and reactive molecules containing the "tag" sequence were affinity purified according to which "tag" sequence was used. dA<sub>22</sub> and dT<sub>22</sub> sequences were isolated on oligo (dT)<sub>12-18</sub> cellulose and oligo (dA)  
 10 cellulose Type 7 (Pharmacia, Piscataway, NJ) respectively. The mixed sequence substrate was annealed to a biotinylated DNA primer complementary to the tag (5' AGCGATCAAGCGATCA-biotin) and captured on streptavidin-agarose. Columns were washed to remove members of the RNA pool that had not reacted with the substrate; ribozymes were eluted with base. Selected populations were  
 15 reverse transcribed with SuperScript II reverse transcriptase (Gibco BRL) using the 3' DNA primer used in PCR amplification (5' GCGACTGGACATCACGAG). The cDNA was then PCR amplified with a 5' DNA primer identical in sequence to the specific substrate used for that round of selection and the usual 3' primer. This DNA was gel-purified on 3% agarose,  
 20 eluted, and used as the input for an additional, regenerative PCR amplification. The DNA was then transcribed, and the resulting RNA was gel-purified on 6% denaturing polyacrylamide, and used as the input for the next round of selection.

### C. D90 pool construction and selection

25 The D90 pool was prepared using the same solid phase DNA synthesis methodologies used to create N90. The D90 sequence was based upon the sequence of clone L1, with the flanking primer-binding sites reproduced exactly and the internal 90 nucleotide region synthesized with a 30% degeneracy (TTCGGTCCAGTGCTCGTG-cac tag gcc gtt cga cca tgt ggg tcc gct gcc agc ggc  
 30 aat ctg gca tgc tat gcg gaa cct tca cat ctt aga cag gag gtt agg

5 tgc-CTCGTGATGTCCAGTCGC, degenerate bases in lowercase). The phosphoramidite ratios again included a correction against the biased incorporation of dG and dT). The degeneracy of the pool was examined by sequencing eight D90 clones. Wild-type bases accounted for 63% of the positions sampled, with non-wild type incorporation of G, A, T, and C being 7.5%, 8.0%, 11.7%, and 9.5% respectively. Synthetic D90 DNA was gel-purified, quantitated, amplified in a 15 mL PCR reaction with standard primers. A RNA pool was transcribed and gel-purified. The selection was carried out in a manner similar to the N90 selection using an input of 332 pmol (2.0 x 10<sup>14</sup> molecules) in the first round and 123 pmol (1.3 x 10<sup>14</sup> molecules) in each of the six subsequent rounds. Reactions were incubated at 25°C for 16 hours in the first and second rounds of selection. As activity increased, the incubation times were steadily decreased to 1 hr, 15 min, 5 min, and 2.5 min in the third, fourth, fifth, and sixth rounds respectively. As a further degree of selection stringency, the concentration of MgCl<sub>2</sub> was reduced to 30 mM in the fourth round and 10 mM in both the fifth and sixth rounds. The most notable differences between this selection and the N90 selection is that only the dA<sub>22</sub> tagged substrate was used and that pool RNA was annealed to primer but was not immobilized on streptavidin during the reaction.

20

#### **D. Isolation of ribozymes**

PCR products from the first, third, fourth, and fifth rounds of the initial selection and from the fifth round of the doped reselection were cloned into the pCR2.1 vector using the TA cloning kit (Invitrogen, Carlsbad, CA) and sequenced using standard dideoxy methods (Sequenase 2.0; USB, Cleveland, OH). Ribozyme templates were amplified from individual clones, transcribed, gel-purified, and assayed individually.

25

**E. Assays for activity**

10 pmol (670 nM final concentration) of  $^{32}\text{P}$  body-labeled RNA was combined with 20 pmol effector (1.3  $\mu\text{M}$ ) in a volume of 5  $\mu\text{L}$ . The mixture was heat-denatured and allowed to anneal by cooling to room temperature. Selection  
5 buffer and  $\text{MgCl}_2$  (60 mM final concentration) were added and the mixture was incubated for 5 min at room temperature. The reactions (15  $\mu\text{L}$  final volume) were initiated by adding 20 pmol (1.3  $\mu\text{M}$ ) substrate oligonucleotide and incubated between 1 min and several days at 25°C. The assay conditions mimicked the conditions used in the selection. The reaction was stopped by the  
10 addition of 3  $\mu\text{L}$  0.5M EDTA and 18  $\mu\text{L}$  2x gel loading buffer (7 M urea, 0.1% bromophenol blue). The reaction components were separated on a 8% denaturing polyacrylamide gel and quantitated with a Molecular Dynamics Phosphorimager.

**F. Mutated ribozyme constructs**

15 Ribozymes containing specific mutations in the 3' primer-binding site were prepared by PCR amplification of L1 DNA templates by primers containing the desired mutations. These primers were often longer than the wild-type primer and extended beyond the original primer-binding site to ensure that the mutation induced mismatch would not adversely affect primer extension. Care was taken  
20 to minimize the amount of starting L1 DNA remaining in the finished constructs. Typically, 1 ng of L1 DNA was used as the input for an initial 100  $\mu\text{L}$  PCR reaction, 1  $\mu\text{L}$  of which was subsequently used to seed a fresh 500  $\mu\text{L}$  reaction. Assuming an average PCR yield of 1 to 5  $\mu\text{g}$  of DNA, less than 1 in  $10^5$  molecules were residual L1 DNA. Each construct was transcribed and the RNA  
25 was gel-purified as previously described.

**G. Ribonuclease digestions**

RNA was dephosphorylated with alkaline phosphatase (Boehringer Mannheim, Indianapolis, IN) and phosphorylated with T4 polynucleotide kinase  
30 (New England Biolabs, Beverly, MA) in the presence of gamma  $^{32}\text{P}$  ATP (7000

Ci / mmol; ICN, Costa Mesa, CA). Digestion reactions were carried out by first heat denaturing 2.5 pmol labeled RNA (170 nM final concentration), 2.5 pmol unlabeled RNA (170 nM), and 10 pmol oligonucleotide effector (670 nM; omitted in some samples) in 5  $\mu$ L water and then cooling to room temperature.

5 Selection buffer and  $MgCl_2$  (60 mM final concentration) were then added, and the reaction was equilibrated for 5 minutes at room temperature. 10 pmol substrate oligonucleotide (670 nM; omitted in some samples) was added, followed by an additional 5 minute equilibration at room temperature before the addition of ribonuclease A (0.002 units; USB) or ribonuclease T1 (2.6 units;

10 USB). Reactions were incubated between 5 min and 1 hour at 25°C, stopped by the addition of 1  $\mu$ g tRNA, phenol/chloroform extracted, and ethanol precipitated. Products were separated on a 8% denaturing polyacrylamide gel and visualized by autoradiography.

## 15 EXAMPLES

### Example 1 *In vitro* selection of a ribozyme ligase

In order to select ribozyme ligases, a scheme similar to that originally employed by Bartel and Szostak<sup>(13)</sup> was utilized, with several important differences. The scheme is shown in Fig. 1: the random sequence pool was only

20 90 residues in length, rather than 220 residues, and a stem-loop structure near the ligation junction was different than that previously employed by Bartel and Szostak<sup>(13)</sup>. After five rounds of selection and amplification, ligation activity was firmly established in the population. Individual ribozymes were cloned and sequenced. As expected based on the increased stringency of the selection, the

25 population during selection collapsed to only a few variants, one of which (L1) predominated which is shown in Fig. 2. The velocity of the selected L1 ribozyme was 0.71 hr<sup>-1</sup> under optimal conditions, roughly as fast as ribozyme ligases initially selected by Bartel and Szostak<sup>(13)</sup> (8 hr<sup>-1</sup>), Hager and Szostak<sup>(14)</sup> (0.4 hr<sup>-1</sup>), and Cuenoud and Szostak<sup>(15)</sup> (3.4 hr<sup>-1</sup>).



The L1 ribozyme was partially mutagenized by carrying out dopping with (37% non-wild type residues) and the resultant population was re-selected for ligation function. After seven cycles of selection and amplification the ligase activity of the population had ceased to improve, and individual ribozymes were  
5 cloned. The best ribozyme from the re-selected population performed 5.6 ligations/hour which is 8-fold better than the parental ribozyme. Comparative sequence analysis revealed that sequence positions in the L1 ribozyme tended to be either highly conserved (22 residues) or highly variant. The probability of finding the L1 ribozyme in a random sequence population was thus estimated to  
10 be  $2 \times 10^{-13}$ , similar to the probability estimated by Ekland *et al.*<sup>16</sup> for finding a Class III ribozyme ligase ( $8 \times 10^{-12}$ ). The L1 ribozyme motif arose independently in the population three times, similar to the number of times the Bartel Class III ribozyme ligase arose.

### 15 **Example 2 Effector dependence of the ribozyme**

The L1 ribozyme was assayed and was found to be far more dependent on its oligonucleotide effector than ribozymes selected by Bartel and Szostak<sup>(13)</sup> which show a 2- to 20-fold dependence of their effector (cDNA process). Extended incubations in the absence of the effector indicated that the background  
20 reaction was more than 10,000-fold slower than in the presence of the effector. This figure favorably compares with typical allosteric enzymes, such as aspartate transcarbamoylase, which are activated only two- to ten-fold by their small molecule effectors<sup>(17)</sup>.

### 25 **Example 3 Determination of the mechanism of allostery**

#### (a) By alignment

Alignment of the re-selected sequences revealed which residues were likely critical for function and identified sequence substitutions that validated a secondary structural model for the active ribozyme (Fig. 2A). The examination  
30 of possible RNA folds in the absence of the oligonucleotide effector immediately

suggested a simple, two-state allosteric model for effector-dependent modulation of ribozyme activity (Fig. 2B). In this model, the primer-binding site can fold over onto the substrate-binding site, much as the pseudosubstrate of a protein enzyme such as a protein kinase can occupy its enzyme active site<sup>(19-21)</sup>. In the presence of a complementary oligonucleotide effector, the primer-binding site assumes an alternative, extended helical conformation and the active site can pair with a RNA substrate. In accord with the precept that evolution is superior to rational design for ribozyme engineering, the selected ribozyme produced as described above exhibited 1,000-fold greater activation by an oligonucleotide effector than a hammerhead ribozyme that was rationally designed to be activated by an oligonucleotide effector<sup>1</sup>.

(b) By detection of inhibitory sequence

If the proposed two-state model for effector-dependent modulation of ligation activity is correct, then deletion of the inhibitory 3' primer-binding site should allow the ribozyme to remain active. To test this hypothesis, a variant of the ribozyme was constructed that lacked the 3' primer-binding site. The deletion variant proved to be extremely active (within three-fold of wild-type in a standard assay).

(c) By sequence substitution

To further validate the two-state model, additional sequence substitutions were introduced into the L1 ribozyme. There are several predicted non-Watson-Crick pairings between ribozyme and substrate, including an A:G pairing at the ligation junction; substrate variants that interact with substrate solely through Watson-Crick pairings are inactive. Similarly, ribozyme variants that disrupt predicted Watson-Crick pairings with the substrate (mut.1 and mut.3 in Fig. 2A) were inactive. However, substrate variants that were predicted to restore Watson-Crick pairings were from 2,000 to 3,000-fold more active with mut.1 and mut.3 ribozymes than was the wild-type substrate. Interestingly, L1

and most other selected ribozyme ligases have chosen to 'invent' substrate-binding sites within their random sequence regions rather than utilize the substrate-binding sites originally designed into their constant regions. Second, sequence substitutions introduced into the oligonucleotide effector

5 (Table I) were again less active (by 100- to 1,000-fold) with the wild-type ribozyme, while compensatory sequence substitutions in the ribozyme's primer-binding site restored both activity and activation. The fact that the degree of activation was smaller with mutant effector:ribozyme pairs than with the wild-type effector and ribozyme is consistent with the structural and mechanistic

10 models we have proposed, since sequence substitutions in the primer-binding site should less efficiently stabilize the "inactive" form of the ribozyme.

Summary of effector mutations on ligase activity. Mutations are shown. Relative activation values indicate the extent to which the effector is able to activate the wild-type L1 ribozyme. Relative induction values refer to the activity

15 of a mutated ribozyme assayed with a complementary effector relative to activity without effector.

Table 1

Effector	Effector sequence	Relative activation	relative induction
wild type	5' GCG ACT GGA CAT CAC GAG	1.0	10000
mut. 1	5' GCG ACT GGA CAT CAC GAA	0.01	130
mut. 3	5' GCG ACT GGA CAT CAC GAC	0.004	100
mut. 4	5' GCG ACT GGA CAT CAC GAT	0.007	160
ac. 1	5' GCG ACT GGG CAG CGC GCA	0.001	130
mut. 2	5' ATA GTC AAG TGC TGT AGG	0.005	>9000

(d) Using nucleases

If the L1 ribozyme is in fact an allosteric enzyme it should be possible to detect the proposed effector-dependent conformational changes. To this end, the ribozyme was probed with nucleases in the presence and absence of its oligonucleotide substrate and effector (Fig. 3). No substrate-dependent changes in nuclease accessibility were identified. However, the patterns of digestion were consistent with the effector-dependent secondary structural models proposed in Fig. 2. For example, G108 and G109 are predicted to lay within a nuclease-labile loop when the primer-binding site folds to form the inactive conformation, and to lay within a nuclease-resistant stem when the effector oligonucleotide is bound to the primer-binding site; G108 and G109 are protected in the presence of effector, and strongly cleaved in its absence. Conversely, G103 and G104 are predicted to lie within a nuclease-resistant stem when the primer-binding site folds to form the inactive conformation. In the presence of the effector, these residues are predicted to lie adjacent to the end of a helix, and thus as the helix "*frays*" these residues may be transiently available to nucleases. In accord with this interpretation, G103 and G104 are strongly protected in the absence of effector, but weakly cleaved in its presence. Other differential cleavages that were observed, such as the protection of U94 in the presence of effector, cannot be readily explained based on the secondary structural models and may reflect allosteric transitions in the tertiary structure of the ribozyme.

**Example 4 Detection of a non-nucleic acid molecule**

Breaker and his co-workers have recently demonstrated that allosteric ribozymes can be generated by appending aptamers (nucleic acid sequences capable of binding proteins) to a hammerhead ribozyme<sup>(2-4)</sup>. The anti-adenosine aptamer was swapped for a segment of the L1 ribozyme that was not conserved during partial randomization and re-selection (Fig. 2; Fig. 5A). The catalytic activity for reporting activation of the ribozyme was ligation. The L1 aptazyme

(i.e. ribozyme containing the aptamer) sensed different concentrations of ATP (Fig. 5B) and was activated up to 30-fold by ATP, a value similar to that observed by Breaker<sup>2</sup> for the hammerhead aptazyme. In order to improve the length and activity composition of the stem structures in the ribozyme and the aptamer were systematically altered and the best construct found designated, L1.dB2-ATPm1 (Fig. 5C), was activated by over 800-fold in the presence of ATP. Given the relative ease with which an oligonucleotide-dependent allosteric ribozyme ligase was converted to a small molecule-dependent allosteric ribozyme ligase, and the ease with which the signaling characteristics of the aptazyme were optimized, it is expected that similar constructs may allow biomedically important effectors such as hormones or neurotransmitters to be detected by ribozymes and the catalytic product of ribozyme activity can be quantitated using off-the-shelf amplification technologies such as PCR, LCR, and 3SR (NASBA)<sup>(22)</sup>.

#### Example 5 Sensitivity of allosteric ribozyme

The activation of the allosteric ribozyme is sensitive to the presence of at least some mismatches as shown in Table 1 above. To determine whether background sequences might yield false positives, the activity of the L1 ribozyme was also assayed in the presence of either a random sequence pool or the cognate oligonucleotide effector mixed with a random sequence pool. Activation was detected only when the cognate oligonucleotide effector was present (data not shown).

The fact that compensatory substitutions in the primer-binding site allow recognition of divergent effectors implies that the L1 ribozyme can be "programmed" to recognize a wider variety of target sequences. In support of this hypothesis, transitions (A for G, G for A; C for T, T for C) were introduced into all but the terminal 3' residue of the oligonucleotide effector (Table 1); this effector could not activate the wild-type L1 ribozyme. Similarly, when a complementary 17-tuple substitution was introduced into the ribozyme

primer-binding site, the ribozyme was not activated by the wild-type effector (<0.01% wild-type activity). However, when the 17-tuple substituted effector was mixed with the 17-tuple substituted ribozyme a 9,000-fold activation was observed, to a level nearly equivalent to that of the wild-type ribozyme:effector pair. The fact that the allosteric activation mechanism lends itself to design and change bodes well for the construction of ribozymes that can sense biomedically or environmentally important sequences. While the mechanism of inhibition of the 17-tuple substituted ribozyme is currently unknown, an inactive conformer distinct from that shown in Figure 2B can be drawn (data not shown).

10

#### **Example 6 Detection using ligation activity and PCR amplification**

In order to determine whether the L1 ribozyme might be used in diagnostic assays, we attempted to detect varying concentrations of the oligonucleotide effector using RT-PCR (Fig. 4). As little as 1 nanomolar (30 femtomoles) of the oligonucleotide effector could be specifically detected, and the amount of effector initially present was closely correlated with the amount of amplification product generated over three orders of magnitude (from 1 nanomolar to 1 micromolar,  $r^2 = 0.98$ ).

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**CLAIMS:**

1. A method for the detection of an analyte in a sample comprising:
  - (i) providing a nucleic acid sequence which is initially essentially catalytically inactive, and which becomes catalytically active in the presence of the analyte;
  - (ii) providing a nucleic acid substrate for the catalytic activity of the nucleic acid sequence;
  - (iii) contacting the nucleic acid sequence and the substrate with the sample under conditions allowing catalytic activity of nucleic acid sequences; thereby allowing the nucleic acid sequence, in the presence of the analyte, to convert the substrate to a catalytic product which is a nucleic acid molecule;
  - (iv) contacting the catalytic product with a nucleic acid amplification system capable of multiplying the number of copies of the catalytic product, a significant increase in the number of copies of the catalytic product indicates the presence of the analyte in the sample.
2. A method according to Claim 1, wherein the nucleic acid amplification system is selected from the group consisting of: polymerase chain reaction (PCR) ligase chain reaction (LCR), 3SR and NASBA.
3. A method according to Claim 1 or 2, wherein the analyte is a nucleic acid sequence.
4. A method according to Claim 1 or 2, wherein the analyte is a non-nucleic acid sequence.
5. A method according to Claim 4, wherein the analyte is selected from the group consisting of: protein, peptide, polypeptide, glycoprotein, glycopeptide hormone, phosphorylated nucleotides, organic or non-organic contaminant, a drug and an infectious organism.
6. A method according to Claim 1, for quantitatively determining the amount of analyte further comprising the following steps:

- (v) determining the amount of amplified catalytic product;
  - (vi) comparing said amount against a predetermined calibration scale correlating the amount of product to the amount of analyte, thereby determining the amount of analyte.
- 5 7. A method according to any one of the preceding claims, wherein the catalytic activity is ligation.

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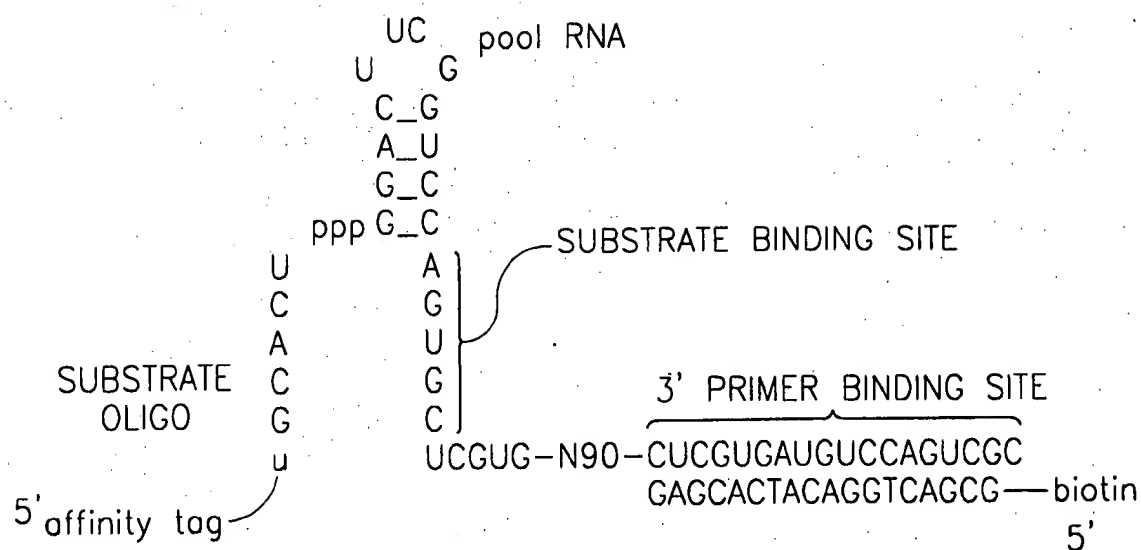
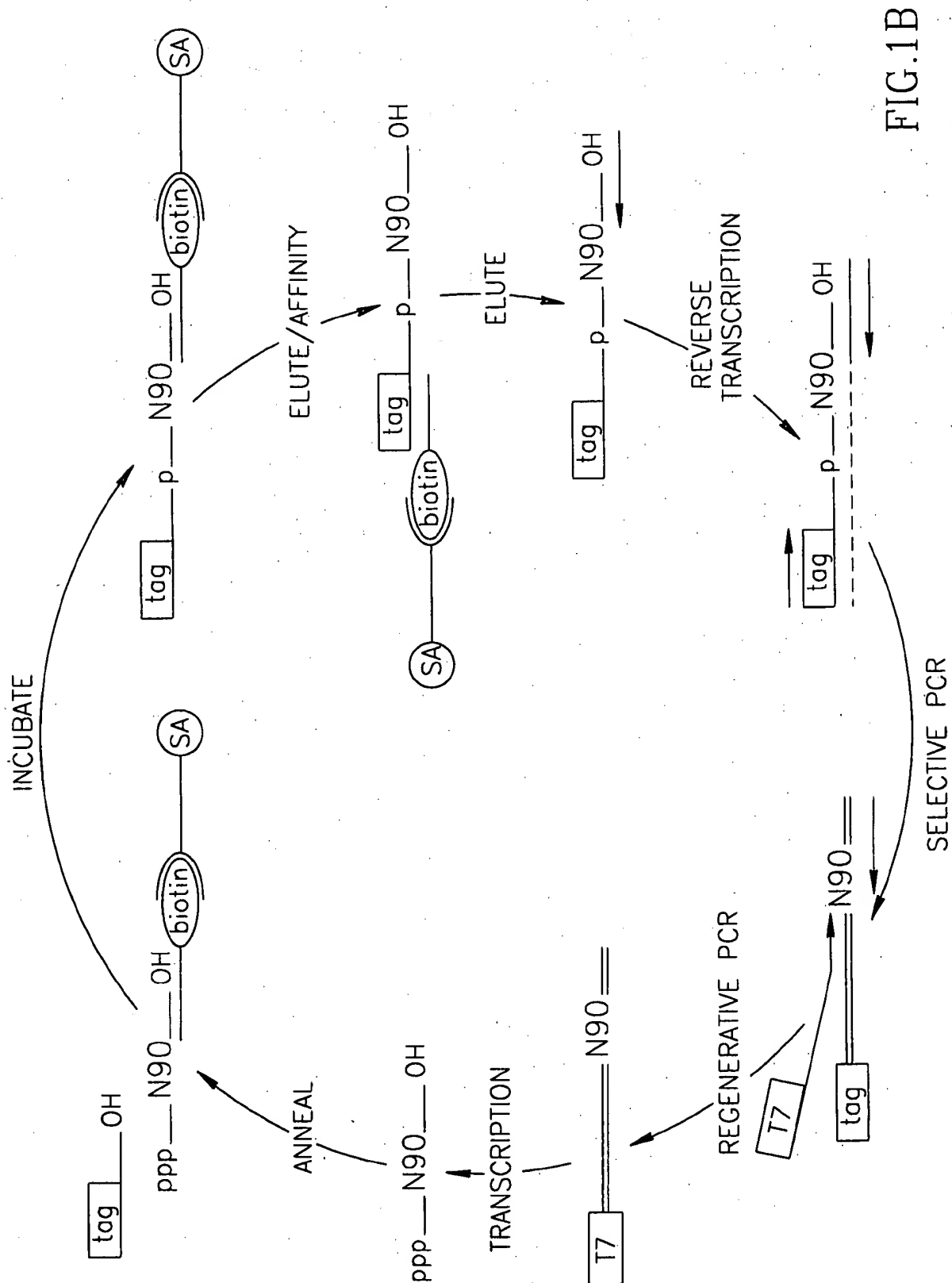


FIG.1A

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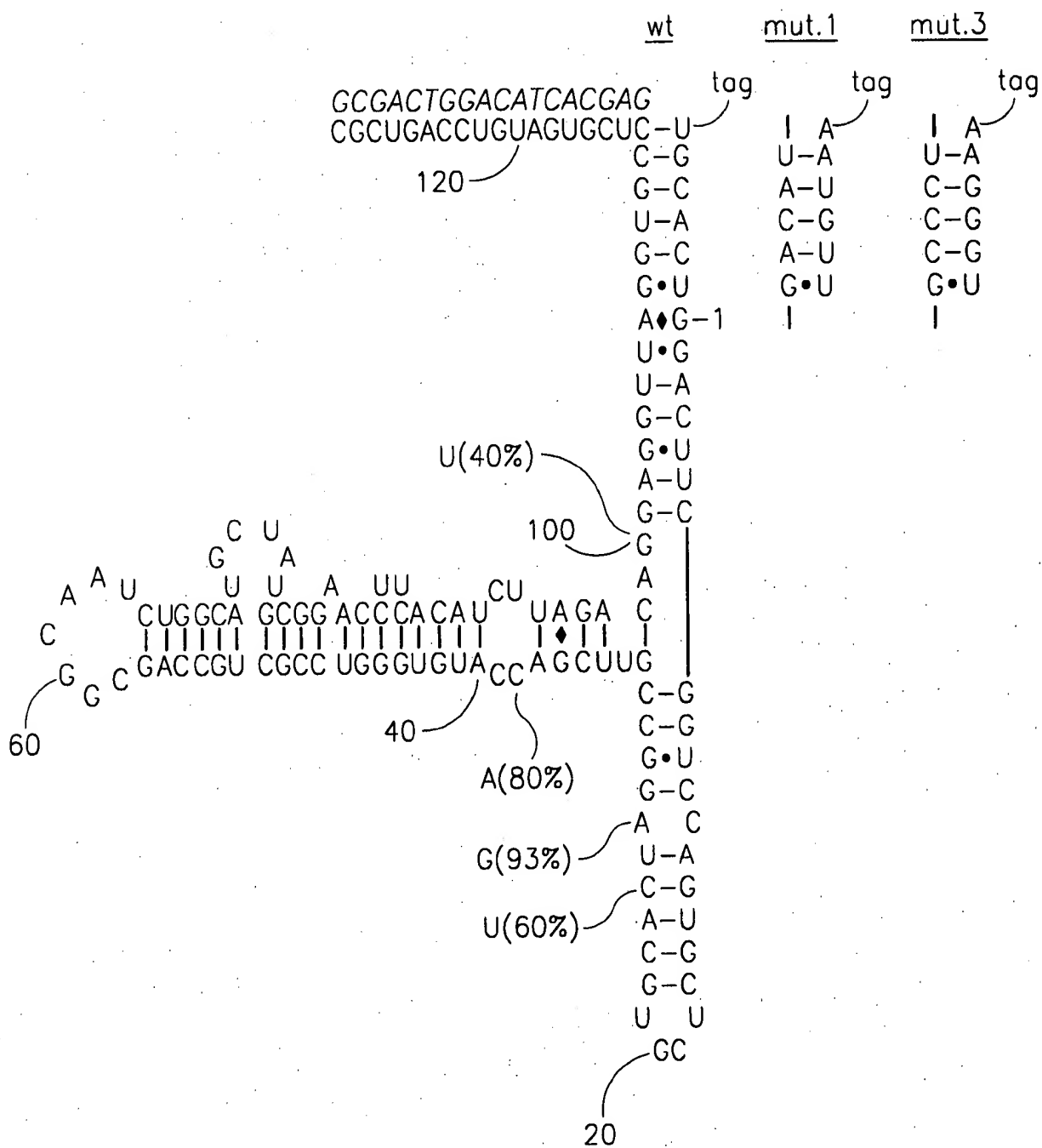


FIG.2A

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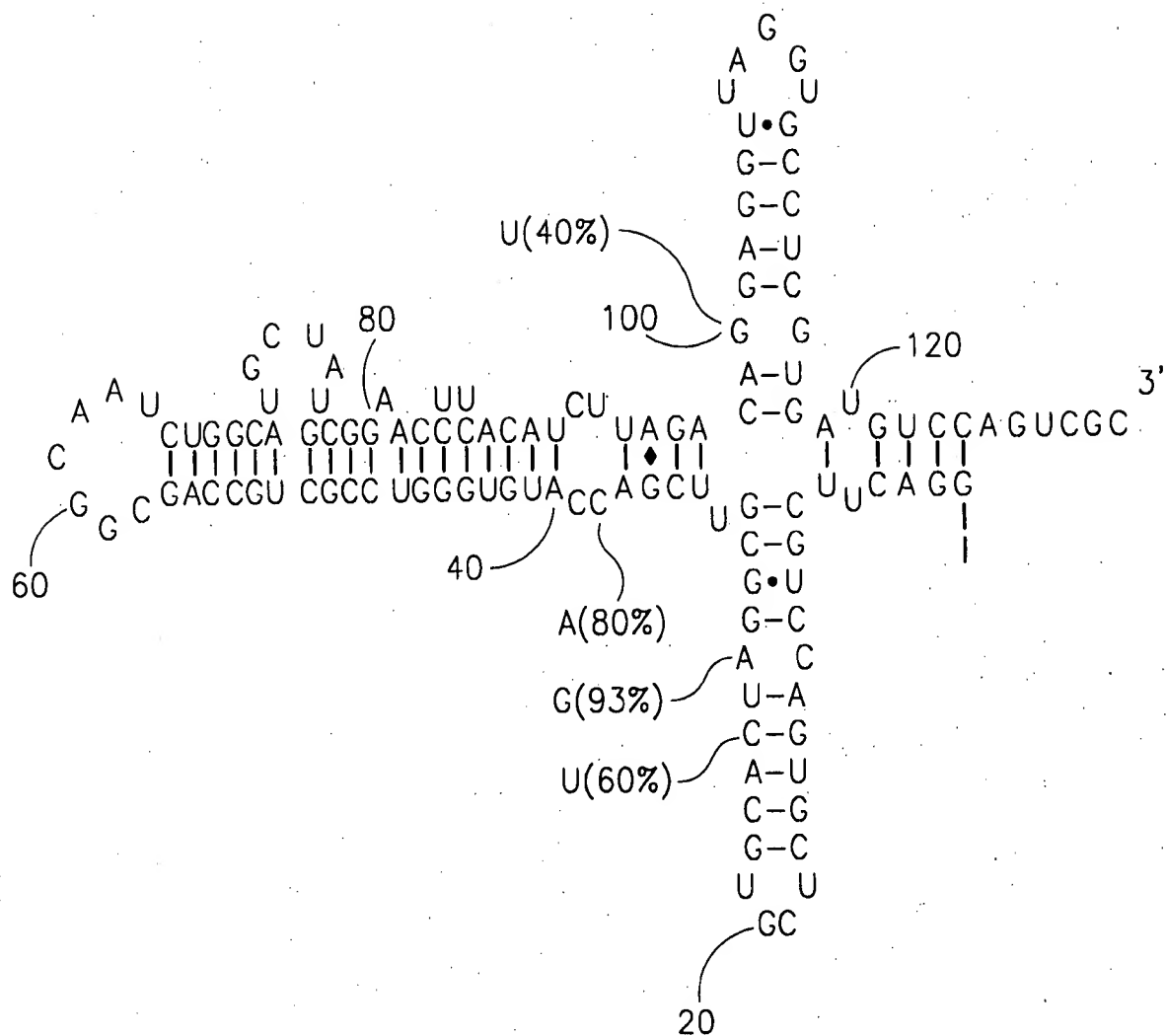


FIG.2B

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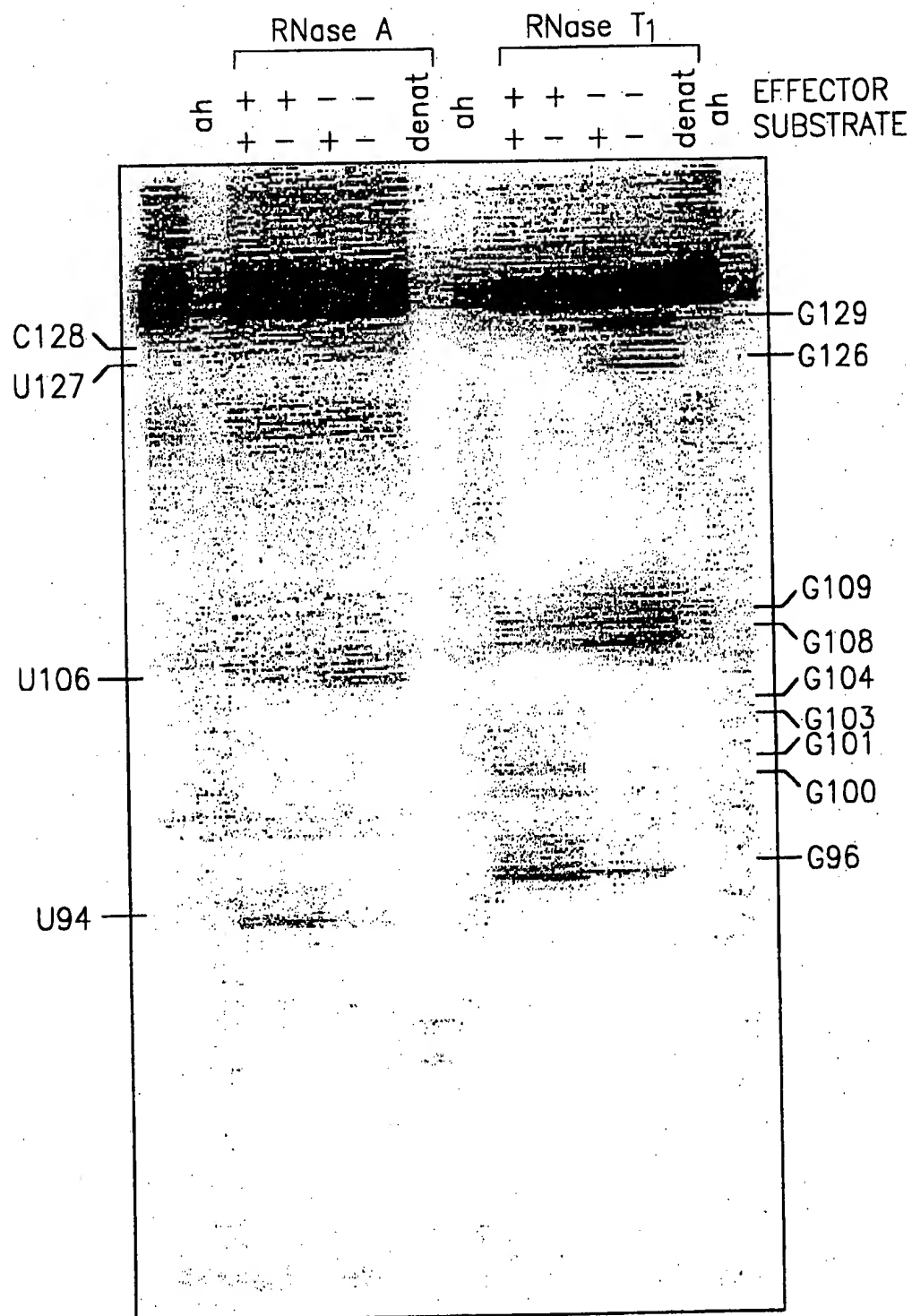


FIG.3

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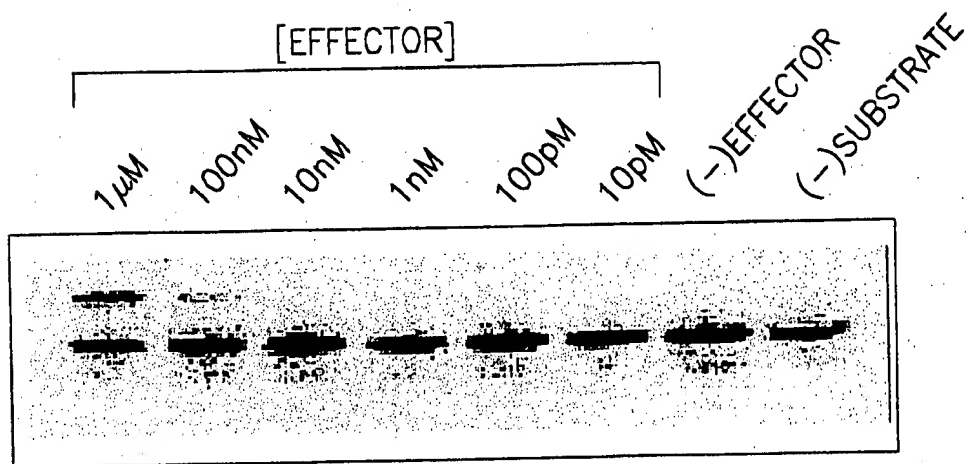


FIG.4A

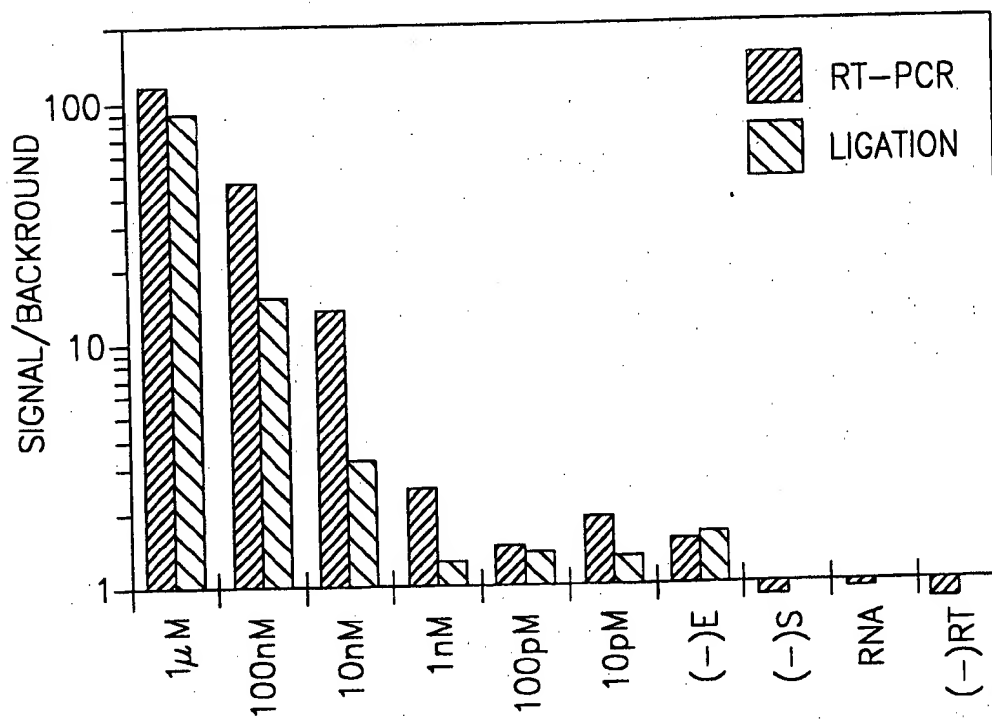


FIG.4B



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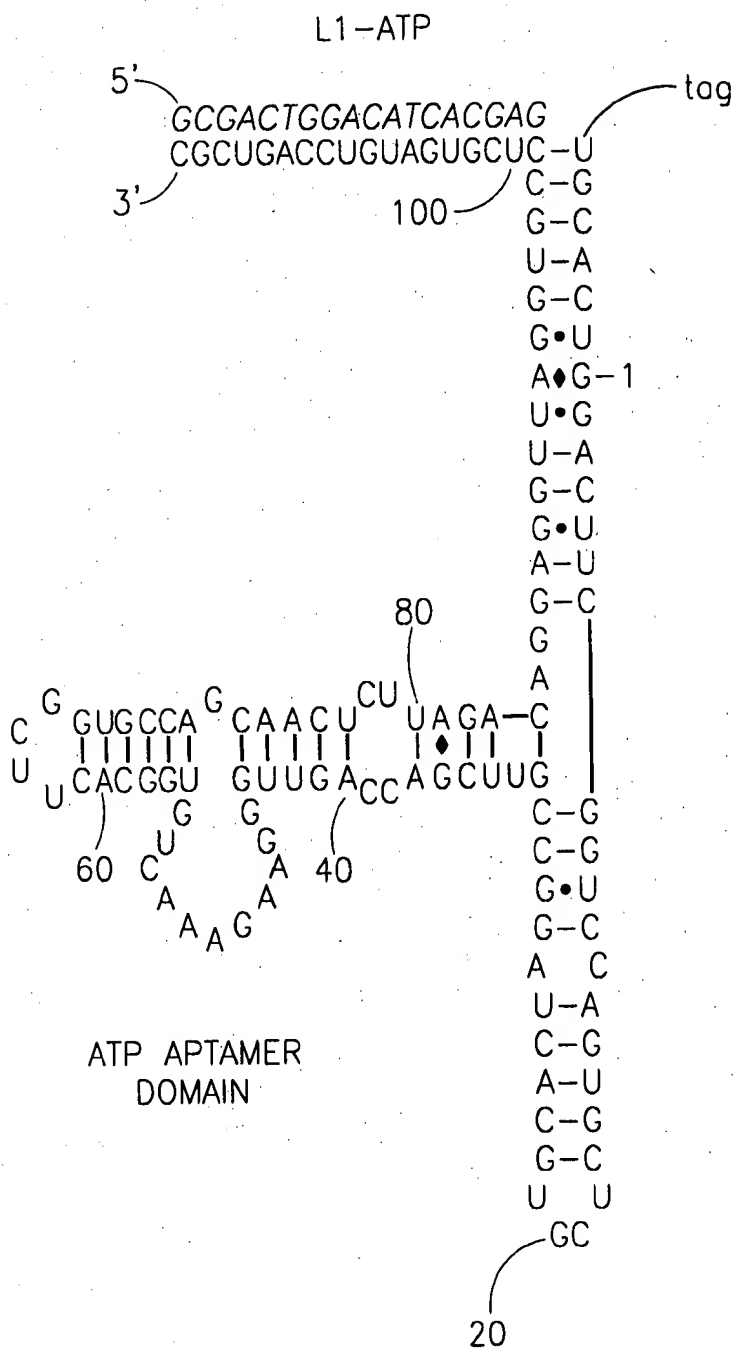


FIG.5A

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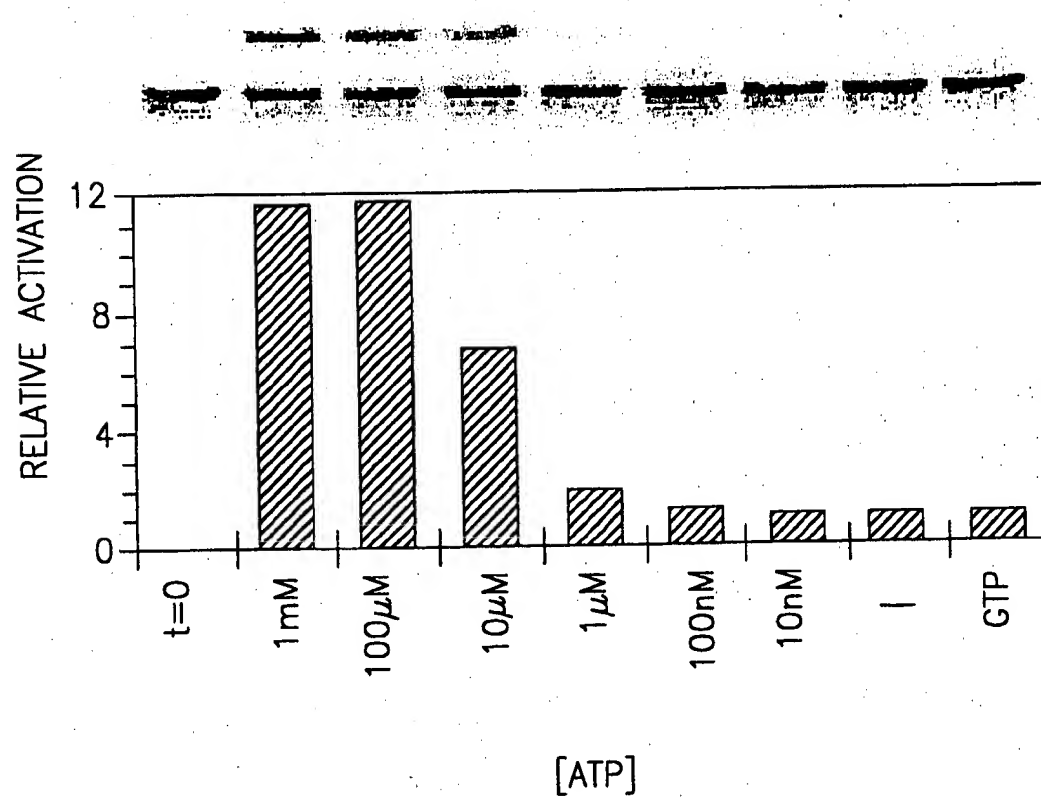


FIG.5B

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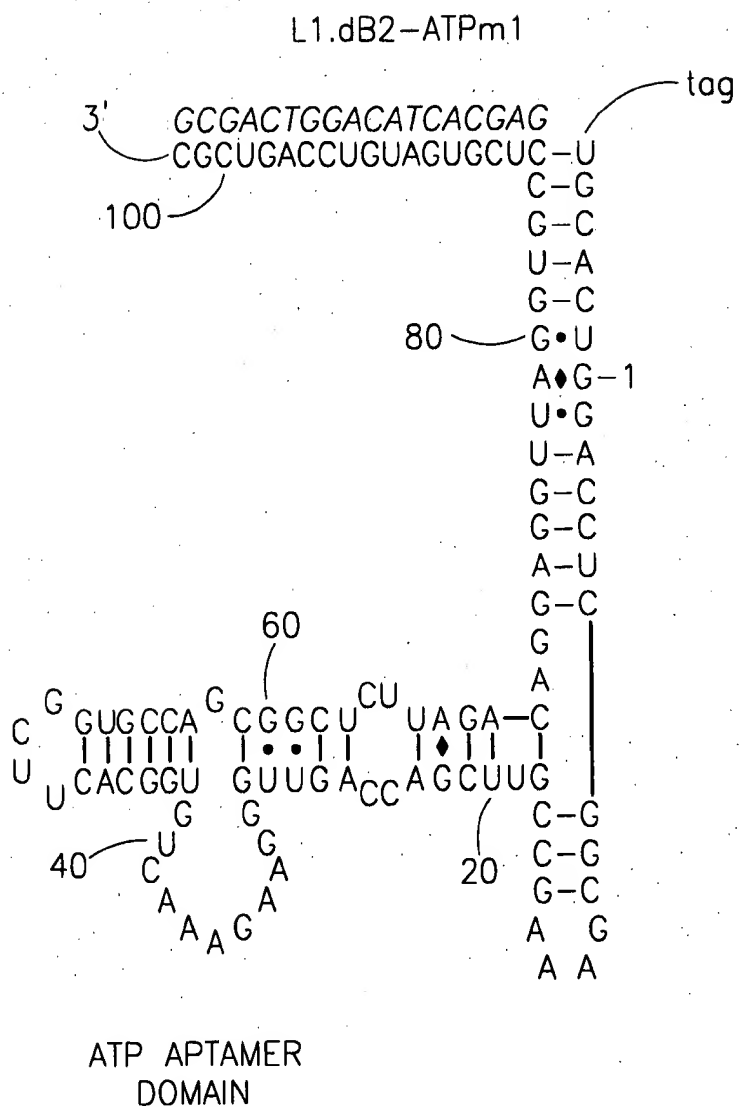


FIG.5C

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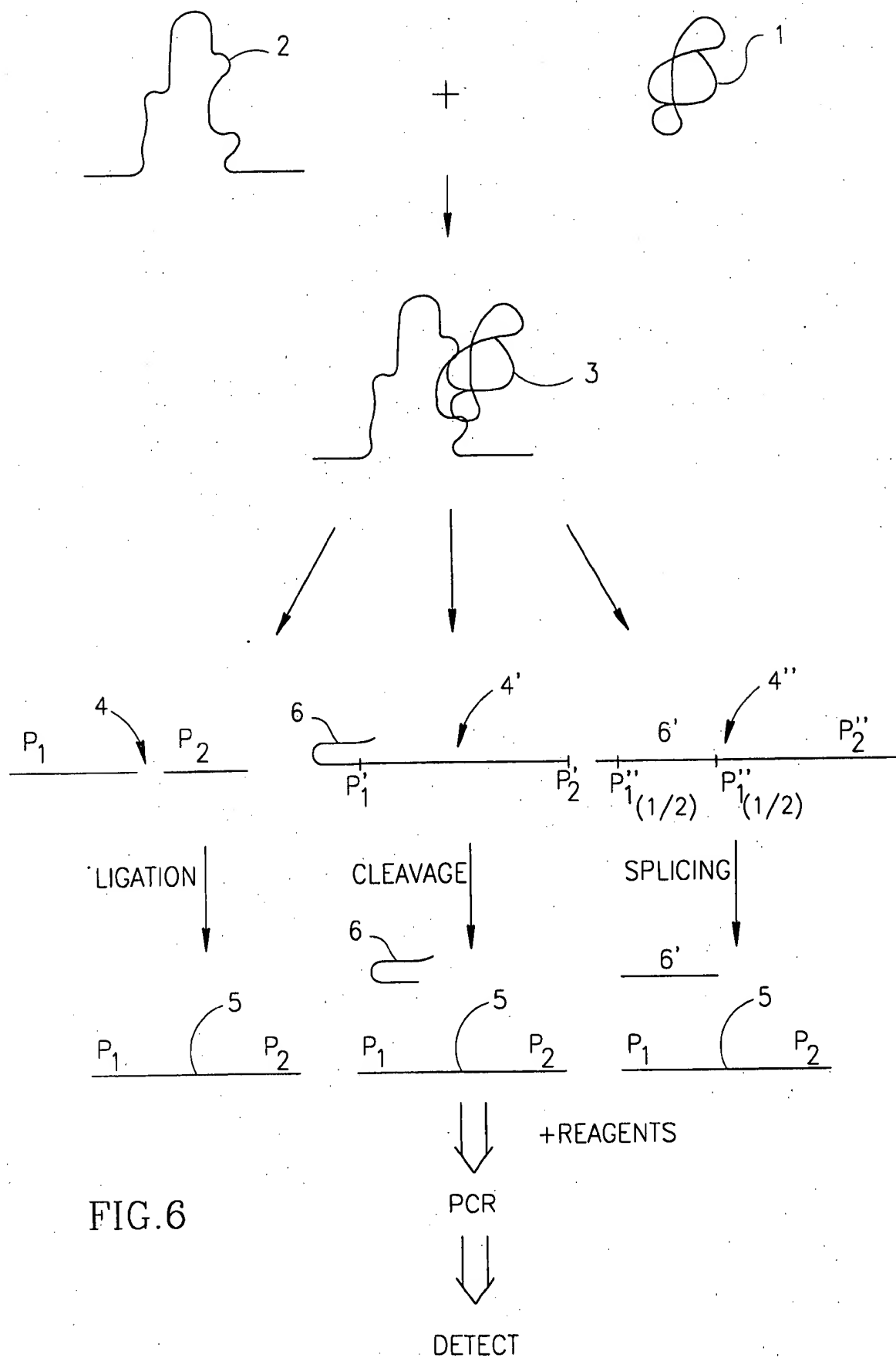


FIG.6